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(57) Abstract

A therapeutic and/or cosmetic formulation comprising at least one anti-sense polynucleotide to a connexin protein together with a pharmaceutically acceptable carrier or vehicle is useful in site specific down regulation of connexin protein expression, particularly in reduction of neuronal cells death, wound healing, reduction of inflammation, decrease of scar formation and skin rejuvenation and thickening.

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FORMULATIONS COMPRISING ANTISENSE NUCLEOTIDES TO CONNEXINS

This invention relates to formulations for use in therapeutic and/or cosmetic treatments, particularly those in which a localised disruption in direct cell-cell communication is desirable.

BACKGROUND

Gap junctions are cell membrane structures which facilitate direct cell-cell communication. A gap junction channel is formed of two hemichannels (connexons), each composed of six connexin subunits. These connexins are a family of proteins, commonly named according to their molecular weight or classified on a phylogenetic basis ie. into an α class and a β class.

An ability to control connexin expression (and in particular to downregulate it) would therefore provide an opportunity to modulate cell-cell communication within a patient for therapeutic and/or remedial purposes. However, as a number of connexin proteins are expressed widely throughout the body, a general downregulatory effect is undesirable in inducing a therapeutic effect at a specific site.

Anti-sense oligodeoxynucleotides (ODN's) have considerable potential as agents for the manipulation of specific gene expression (reviewed: Stein *et al.*, 1992; Wagner 1994). However, there remain difficulties which need to be overcome. These include the short half life of such ODN's (unmodified phosphodiester oligomers typically have an intracellular half life of only 20 minutes owing to intracellular nuclease degradation (Wagner 1994)) and their delivery consistently and reliably to target tissues.

It was with the intent of at least partially overcoming these difficulties that the applicants devised the present invention.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect, the invention provides a formulation for use in therapeutic and/or cosmetic treatment, which formulation comprises:

at least one anti-sense polynucleotide to a connexin protein; together with a pharmaceutically acceptable carrier or vehicle.

In one preferred form, the formulation contains polynucleotides to one connexin protein only. Most preferably, this connexin protein is connexin 43.

Many aspects of the invention are described with reference to oligodeoxynucleotides. However it is understood that other suitable polynucleotides (such as RNA polynucleotides) may be used in these aspects.

Alternatively, the formulation contains oligodeoxynucleotides to more than one connexin protein. Preferably, one of the connexin proteins to which oligodeoxynucleotides are directed is connexin 43. Other connexin proteins to which oligodeoxynucleotides are directed include connexin 26, connexin 31.1 and connexin 32.

Conveniently, the oligodeoxynucleotide to connexin 43 is selected from:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;
GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

Most conveniently, the oligodeoxynucleotide to connexin 43 is:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC.

Conveniently, the oligodeoxynucleotide to connexin 26 is:

TCC TGA GCA ATA CCT AAC GAA CAA ATA.

Conveniently, the oligodeoxynucleotide to connexin 31.1 is:

CGT CCG AGC CCA GAA AGA TGA GGT C.

Conveniently, the oligodeoxynucleotide to connexin 32 is:

TTT CTT TTC TAT GTG CTG TTG GTG A.

The anti-sense polynucleotides may be formulated for parenteral, intramuscular, intracerebral, intravenous, subcutaneous or transdermal administration. The antisense polynucleotides are preferably administered topically (at the site to be treated). Suitably the antisense polynucleotides are combined with a pharmaceutically acceptable carrier, vehicle or diluent to provide a pharmaceutical composition.

Suitable pharmaceutically acceptable carriers or vehicles include any of those commonly used for topical administration. The topical formulation may be in the form of a cream, ointment, gel, emulsion, lotion or paint. The formulation of the invention may also be presented in the form of an impregnated dressing.

Suitable carrier materials include any carrier or vehicle commonly used as a base for creams, lotions, gels, emulsions, lotions or paints for topical administration. Examples include emulsifying agents, inert carriers including hydrocarbon bases, emulsifying bases, non-toxic solvents or water-soluble bases. Particularly suitable examples include lanolin, hard paraffin, liquid paraffin, soft yellow paraffin or soft white paraffin, white beeswax, yellow beeswax, cetostearyl alcohol, cetyl alcohol, dimethicones, emulsifying waxes, isopropyl myristate, microcrystalline wax, oleyl alcohol and stearyl alcohol.

Preferably, the pharmaceutically acceptable carrier or vehicle is a gel, suitably a nonionic polyoxyethylene-polyoxypropylene copolymer gel, for example, a Pluronic gel, preferably Pluronic F-127 (BASF Corp.). This gel is particularly preferred as it is a liquid at low temperatures but rapidly sets at physiological temperatures, which confines the release of the ODN component to the site of application or immediately adjacent that site.

An auxiliary agent such as casein, gelatin, albumin, glue, sodium alginate, carboxymethylcellulose, methylcellulose, hydroxyethylcellulose or polyvinyl alcohol may also be included in the formulation of the invention.

The pharmaceutical composition may be formulated to provide sustained release of the antisense polynucleotide.

Conveniently, the formulation further includes a surfactant to assist with oligodeoxynucleotide cell penetration or the formulation may contain any suitable loading agent. Any suitable non-toxic surfactant may be included, such as DMSO. Alternatively a transdermal penetration agent such as urea may be included.

In a further aspect, the invention provides a method of site-specific downregulation of connexin protein expression for a therapeutic and/or cosmetic purpose which comprises administering a formulation as defined above to a site on or within a patient at which said downregulation is required.

In still a further aspect, the invention provides a method of reducing neuronal cell death which would otherwise result from a neuronal insult to a specific site in the brain, spinal cord or optic nerve of a patient which comprises the step of administering a formulation as defined above to said site to downregulate expression of connexin protein(s) at and immediately adjacent said site.

Preferably, the formulation is administered to reduce neuronal loss due to physical trauma to the brain, spinal cord or optic nerve.

Conveniently, the formulation is administered in a sufficient amount to downregulate expression of said connexin protein(s) for at least 24 hours post-administration.

In yet a further aspect, the invention provides a method of promoting wound healing in a patient which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

Usually, the wound will be the result of trauma, including burns. It may however be the result of surgery.

In yet a further aspect, the invention provides a method of reducing

inflammation as part of treating a wound and/or tissue subjected to physical trauma which comprises the step of administering a formulation as defined above to or proximate to said wound or tissue.

Preferably, said wound is a burn.

Alternatively, said wound is the result of physical trauma to tissue, including neuronal tissue such as the brain, spinal cord or optic nerve.

In yet a further aspect, the invention provides a method of decreasing scar formation in a patient who has suffered a wound which comprises the step of administering a formulation as defined above to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

Again, the wound may be the result of trauma or surgery, with the formulation being applied to the wound immediately prior to surgical repair and/or closure thereof.

In yet a further aspect, the invention provides a method of skin rejuvenation or thickening for a cosmetic or therapeutic purpose which comprises the step of administering, once or repeatedly, a formulation as defined above to the skin surface.

Conveniently, said formulation includes oligodeoxynucleotides directed to connexin 26 or connexin 43 and is administered to regulate epithelial basal cell division and growth.

In another embodiment, said formulation includes oligodeoxynucleotides directed to connexin 31.1 and is administered to regulate outer layer keratinisation.

Preferably, the formulation is a cream or gel.

DESCRIPTION OF THE DRAWINGS

Figures 1 to 5 show sections of rat brain lesions treated with Pluronic gel containing antisense oligodeoxynucleotides specific to connexin 43, or for control lesions, Pluronic gel alone. In all cases lesions were sectioned serially in a coronal plane and the mid point sections used for analysis. Each image (except Figure 5) shows 4 mm by 5.33 mm of tissue. Figure 5 is approximately 1.2 mm by 2 mm.

Figure 1: Figures 1A and 1C show two side of a control lesion 24 hours after

lesioning. The lesion has been treated with Pluronic gel alone. The sections have been Nissl stained (blue nuclei) and antibody labelled with the neuronal marker Neuronal-N (brown cells). Figures 1B and 1D show grey scale images of 1A and 1C respectively with the outline of the lesion marked. Note the large size of the lesion and the irregular spreading edges. The lesion has spread downwards toward the corpus callosum (dashed line) within just 24 hours of lesioning.

Figure 2: A control lesion 24 hours after wounding. Figure 2A shows Nissl staining (blue nuclei) and Neuronal-N labelling of viable neurons. Figure 2B is a grey scale equivalent with the lesion edge marked and the top of the corpus callosum marked (dashed line). The original needle tract is clear but neuronal death has occurred well back from the lesion edge as indicated by the Neuronal-N labelling. The edges of the lesion are irregular and the lesion, within just 24 hours, has spread right down into the corpus callosum.

Figure 3: Figures 3A and 3B are colour and grey scale images of a connexin 43 antisense treated lesion, 48 hours after lesioning. The lesion outline has been marked on Figure 3B to show the extent of the lesion and the top of the corpus callosum marked (dashed line). Figure 3A has been stained for Nissl (blue nuclei) and Neuronal-N (pink cells). Note how compact the lesion is, even after 48 hours, compared with control lesions (Figures 1 and 2). While there is some spread to the right hand side, the left side of the lesion essentially follows the original needle tract with little sign of spreading. The left side of the lesion is very straight and it has not spread down to the corpus callosum.

Figure 4: Figures 4A and 4B show another connexin 43 antisense treated lesion 48 hours after wounding. The labelling is the same as in Figure 3 with the lesion outlined on the grey scale image (Figure 4B). Even after 48 hours this lesion is extremely compact with slight spreading only to the left (medial side). Note how straight the right hand side of the lesion is with viable neurons right up to the edge of the needle tract (and indeed surviving within the lesioned area). The lesion is well above the corpus callosum (dashed line) indicating virtually no downward spread.

Figure 5: A higher magnification view showing the edge of a connexin 43

antisense treated lesion. The edge of the lesion has been marked showing viable neurons (Neuronal-N labelled) right up to the edge of the wounding needle tract even 48 hours after lesioning.

Figure 6: GFAP (red) and connexin43 (green) immunohistochemical labelling of a connexin43 specific antisense treated lesion, 24 hours after lesioning. The image is taken at the lateral edge of the lesion at a point half way down the depth of the lesion. Activated astrocyte levels are elevated compared with controls (Figure 7) and connexin 43 levels are markedly reduced. The connexin labelling remaining is generally associated with blood vessels (arrows).

Figure 7: GFAP (red) and connexin43 (green) immunohistochemical labelling of a control lesion, 24 hours after lesioning. The image is from the medial edge of the lesion and shows GFAP levels slightly elevated over unlesioned cortex. Note the extensive connexin 43 labelling, often co- localised with the GFAP astrocytic marker (arrows).

Figure 8 shows a comparison of lesion cross sectional lower half areas 24 hours (circles) and 48 hours (diamonds) after lesioning. The analysis was carried out on a mid section of serially sectioned lesion cut on the coronal plane. Lesions were assessed using Neuronal-N antibody labelling to delineate viable neurons. DB1 treated lesions (green markers) have been treated with antisense oligodeoxynucleotides specific to connexin 43. The gel only lesion group (red markers) also includes empty lesions while the HB3 group (purple markers) are treated with gel containing random sequence control oligodeoxynucleotides. Note that while connexin 43 antisense treated lesions can be large (presumably where the antisense has not been well delivered), the smallest lesions are all connexin 43 antisense treated. Lesions were made to a depth of 2 mm and analysis covers 1 mm and below so as to exclude the outer edge where the antisense did not sit.

Figure 9: Lesions in rat spinal cord 24 hours after treatment with connexin 43 sense and antisense ODN's. The sense lesions were no different from untreated controls whereas the antisense treated lesions were smaller and with reduced inflammation.

Figure 10: Lesions in neonatal mouse fore paws 24 hours after treatment with connexin 43 sense ODNs (left paw) or antisense ODNs (right paw). Note the reduction in inflammation and increased rate of healing on the antisense treated paw.

Figure 11: Sections through the centre of the 24 hour wounds shown in Figure 10. The sections have been stained with toluidine blue to reveal neutrophils. There are significantly less neutrophils in the antisense treated wound which was also less inflamed.

Figure 12: Pairs of rat paw lesions five days after lesioning that have been treated with connexin 43 specific antisense ODNs or sense control ODNs. Antisense treated lesions are healing quicker and show less signs of scarring.

Figure 13: Pairs of rat paw lesions made at the neonate stage, and viewed here 8 days after lesioning. Lesions were treated with connexin 43 specific antisense or control sense ODN. Hair has grown and it is clear that antisense treatment has resulted in smaller scars and less hair loss. The site of the lesion remains prominent in the sense treated control but is difficult to detect in the antisense treated limb.

DESCRIPTION OF THE INVENTION

As broadly defined above, the focus of the invention is on site-specific downregulation of connexin expression. This will have the effect of reducing direct cell-cell communication at the site at which connexin expression is downregulated, which gives rise to numerous therapeutic/cosmetic applications as described below.

The downregulation of connexin expression is based generally upon the anti-sense approach using antisense polynucleotides (such as DNA or RNA polynucleotides), and more particularly upon the use of antisense oligodeoxynucleotides (ODN). These polynucleotides (eg. ODN) target the connexin protein(s) to be downregulated. Typically the polynucleotides are single stranded, but may be double stranded.

The antisense polynucleotide may inhibit transcription and/or translation of the connexin. Preferably the polynucleotide is a specific inhibitor of transcription and/or translation from the connexin gene, and does not inhibit transcription and/or

translation from other genes. The product may bind to the connexin gene or mRNA either (i) 5' to the coding sequence, and/or (ii) to the coding sequence, and/or (iii) 3' to the coding sequence.

Generally the antisense polynucleotide will cause the expression of connexin mRNA and/or protein in a cell to be reduced.

The antisense polynucleotide is generally antisense to the connexin mRNA. Such a polynucleotide may be capable of hybridising to the connexin mRNA and may thus inhibit the expression of connexin by interfering with one or more aspects of connexin mRNA metabolism including transcription, mRNA processing, mRNA transport from the nucleus, translation or mRNA degradation. The antisense polynucleotide typically hybridises to the connexin mRNA to form a duplex which can cause direct inhibition of translation and/or destabilisation of the mRNA. Such a duplex may be susceptible to degradation by nucleases.

The antisense polynucleotide may hybridize to all or part of the connexin mRNA. Typically the antisense polynucleotide hybridizes to the ribosome binding region or the coding region of the connexin mRNA. The polynucleotide may be complementary to all of or a region of the connexin mRNA. For example, the polynucleotide may be the exact complement of all or a part of connexin mRNA. However, absolute complementarity is not required and polynucleotides which have sufficient complementarity to form a duplex having a melting temperature of greater than 20°C, 30°C or 40°C under physiological conditions are particularly suitable for use in the present invention.

Thus the polynucleotide is typically a homologue of the mRNA. The polynucleotide may be a polynucleotide which hybridises to the connexin mRNA under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

The polynucleotide will typically be from 6 to 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length. The polynucleotides may be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 1000, 2000 or 3000 or more nucleotides in length.

The connexin protein or proteins targeted by the ODN will be dependent upon the site at which downregulation is to be effected. This reflects the non-uniform make-up of gap junction(s) at different sites throughout the body in terms of connexin sub-unit composition. The connexin may be any connexin that naturally occurs in a human or animal. The connexin gene (including coding sequence) generally has homologue with any of the specific connexins mentioned herein, such as homology with the connexin 43 coding sequence shown in Table 1. The connexin is typically an α or β connexin. Preferably the connexin is expressed in the skin or nervous tissue (including brain cells).

Some connexin proteins are however more ubiquitous than others in terms of distribution in tissue. One of the most widespread is connexin 43. ODN's targeted to connexin 43 are therefore particularly suitable for use in the present invention.

It is also contemplated that ODN's targeted at separate connexin proteins be used in combination (for example 1, 2, 3, 4 or more different connexins may be targeted). For example, ODN's targeted to connexin 43, and one or more other members of the connexin family (such as connexin 26, 31.1, 32, 36, 40 and 45) can be used in combination.

Individual antisense polynucleotides may be specific to a particular connexin, or may target 1, 2, 3 or more different connexins. Specific polynucleotides will generally target sequences in the connexin gene or mRNA which are not conserved between connexins, whereas non-specific polynucleotides will target conserved sequences.

The ODN's for use in the invention will generally be unmodified phosphodiester oligomers. They will vary in length but with a 30 mer ODN being particularly suitable.

The antisense polynucleotides may be chemically modified. This may enhance their resistance to nucleases and may enhance their ability to enter cells. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates,

N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides.

The precise sequence of the antisense polynucleotide used in the invention will depend upon the target connexin protein. For connexin 43, the applicant's have found ODN's having the following sequences to be particularly suitable:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;
GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT

ODN's directed to other connexin proteins can be selected in terms of their nucleotide sequence by any convenient, and conventional, approach. For example, the computer programmes MacVector and OligoTech (from Oligos etc. Eugene, Oregon, USA) can be used. For example, ODN's for connexins 26, 31.1 and 32 have the following sequences:

5' TCC TGA GCA ATA CCT AAC GAA CAA ATA (connexin 26)
5' CGT CCG AGC CCA GAA AGA TGA GGT C (connexin 31.1)
5' TTT CTT TTC TAT GTG CTG TTG GTG A (connexin 32)

Once selected, the ODN's can be synthesised using a DNA synthesiser.

For use in the invention, the ODN(s) require site-specific delivery. They also require delivery over an extended period of time. While clearly the delivery period will be dependent upon both the site at which the downregulation is to be

induced and the therapeutic effect which is desired, continuous delivery for 24 hours or longer will often be required.

In accordance with the present invention, this is achieved by inclusion of the ODN(s) in a formulation together with a pharmaceutically acceptable carrier or vehicle, particularly in the form of a formulation for topical administration.

Once prepared, the formulations of the invention have utility in any therapeutic/cosmetic approach where a transient and site-specific interruption in cell-cell communication is desirable. These include in treating neuronal damage in the brain, spinal cord or optic nerve (where the damage is to be localised as much as possible), in the promotion of wound healing and in reducing scar formation following, for example, cosmetic surgery or burns.

In particular, topical formulations such as creams can be employed to regulate epithelial basal cell division and growth (using ODN's targeted to connexin 43) and outer layer keratinisation (using ODN's targeted to connexin 31.1).

The antisense polynucleotides (including the ODN) may be present in a substantially isolated form. It will be understood that the product may be mixed with carriers or diluents which will not interfere with the intended purpose of the product and still be regarded as substantially isolated. A product of the invention may also be in a substantially purified form, in which case it will generally comprise 90%, e.g. at least 95%, 98% or 99% of the polynucleotide or dry mass of the preparation.

Administration

The antisense polynucleotides (including ODN's) of the invention (typically in the form of the formulation discussed herein) may thus be administered to a human or animal in need of treatment, such as a human or animal with any of the diseases or conditions mentioned herein. The condition of the human or animal can thus be improved. The polynucleotide and formulation may thus be used in the treatment of the human or animal body by therapy. They may be used in the manufacture of a medicament to treat any of the conditions mentioned herein.

The antisense polynucleotides may be administered by typically (at the site

to be treated). Preferably the antisense polynucleotides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intracerebral, intravenous, subcutaneous, or transdermal administration.

The dose at which an antisense polynucleotide is administered to a patient will depend upon a variety of factors such as the age, weight and general condition of the patient, the condition that is being treated, and the particular antisense polynucleotide that is being administered. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight.

Uptake of nucleic acids by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. The formulation which is administered may contain such agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™).

The routes of administration and dosages described above are intended only as a guide since a skilled physician will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

Homologues

Homology and homologues are discussed herein (eg. the polynucleotides may be a homologue of sequence in connexin mRNA). Such polynucleotides typically have at least 70% homology, preferably at least 80, 90%, 95%, 97% or 99% homology with the relevant sequence, for example over a region of at least 15, 20, 40, 100 more contiguous nucleotides (of the homologous sequence).

Homology may be calculated based on any method in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to

calculate homology or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The homologous sequence typically differs from the relevant sequence by at least (or by no more than) 2, 5, 10, 15, 20 more mutations (which may be substitutions, deletions or insertions). These mutations may be measured across any of the regions mentioned above in relation to calculating homology.

The homologous sequence typically hybridises selectively to the original sequence at a level significantly above background. Selective hybridisation is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C). However, such hybridisation may be carried out under any suitable conditions known in the art (see Sambrook *et al* (1989), Molecular Cloning: A Laboratory Manual). For example, if high stringency is required, suitable conditions include 0.2 x SSC at 60°C. If lower stringency is required, suitable conditions include 2 x SSC at 60°C.

Various aspects of the invention will now be described with reference to the following experimental section which will be understood to be provided by way of illustration only and not to constitute a limitation on the scope of the invention.

EXPERIMENTAL

EXPERIMENT 1

MATERIALS AND METHODS

Antisense application

30% Pluronic F-127 gel (BASF Corp) in phosphate buffered saline (molecular grade water) was used to deliver unmodified a1 connexin (connexin 43) specific anti-sense ODN's to the developing chick embryo (Simons, *et al.*, 1992). Chick embryos were incubated at 38°C and staged according to Hamilton and Hamburger stages. Eggs were windowed and the vitleline and amniotic membranes over the area to be treated were opened using fine forceps. After anti-sense application eggs were sealed with tape and replaced in the incubator for 48 hours at which time most experiments were analysed, the exception being for the time course analysis of a1 connexin "knockdown" and recovery.

Pluronic gel is liquid at low temperatures, 0-4°C, but sets when dropped onto the embryo at physiological temperature, remaining in place for at least 12 hours. The gel has the additional advantage of being a mild surfactant and this, used either alone or in conjunction with DMSO, appeared to markedly expedite ODN penetration into cells (Wagner, 1994). Addition of an FITC tag to DB1 ODN, viewed using confocal laser scanning microscopy, demonstrated intracellular penetration of the probes. Sequences of deoxyoligonucleotides used are shown in Table 1.

Table 1: The Effect on Limb Development of ODN Application Between Stages 8 & 14 of Chick Embryo Development

Antisense oligodeoxynucleotides to Connexin 43
DB1 GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC
CG1 GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT
Control oligodeoxynucleotides
DB1(sense) GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC
DB1(chick) GTA GTT ACG ACA GGA GGA ATT GTT CTC GTC
CV3(random) TCG AAC TGT CAA GAC TGC TAT GGC GAT CAT
Gel only

All ODN's were applied at 0.5-1.0 mM final concentration following dose dependent analysis during preliminary experiments covering a range of concentrations from 0.05 mM to 50 mM. General toxicity effects only became apparent with ODN concentrations greater than 10 mM. ODN gel mixtures were prepared from concentrated stock solutions stored at -80°C.

Anti-sense sequences

DB1 is a mouse anti-sense sequence, complementary to bases 1094 - 1123 of the a1 connexin gene. It has four mismatches with chick a1 connexin sequence. CG1 is complementary to chick a1 connexin bases 720-749. Efficacy of this probe was improved with 1% Dimethylsulphoxide (DMSO) added to the gel. DMSO had no added effect on other anti-sense ODN or control results.

Control sequences

DB1(Chick) is the chick a1 connexin equivalent of DB1 matching chick a1 connexin bases 954-983. Analysis however, indicates a high probability of forming stem loop structures (G = -7.0 kcal/mol, Loop Tm = 92°) and homodimerisation (Tm = 1.5°) and therefore acts as a control sequence. It has been reported that some sense oligonucleotides can form stable DNA triplets (Neckers *et al.*, 1993) inhibiting transcription. However, this was not apparent with DB1 (sense). A random control sequence with no stable secondary structure (G = 1.4 kcal/mol) and unstable

homodimerisation was also used, called CV3. An additional control applying equal concentration mixture of DB1 and DB1 (sense) gave background levels of defects.

Monitoring of protein knockdown

Immunohistochemical localisation of a1 connexin gap junction protein at cell-cell interfaces provides a direct measurement of the anti-sense effect. Anti-peptide a1 connexin specific antibody probes were used to stain wholemount embryos and the connexin distribution was analysed using confocal laser scanning microscopy according to established procedures (Green *et al.*, 1995). Control labelling for two other connexins expressed in the developing chick embryo (connexins b1 & b2) was similarly carried out, also using sequence specific antibodies (Becker *et al.*, 1995).

RESULTS

Reduction of a1 connexin expression

Using Pluronic F-127 gel to deliver unmodified a1 connexin specific anti-sense ODN's to the developing chick embryo, protein expression can be interfered with at chosen time points and allows the anti-sense treatment to be targeted to specific regions of a chick embryo. A droplet of gel containing the anti-sense at a relatively low concentration was placed precisely onto individual embryos. The gel sets and remains in place for at least 12 hours and thus a sustained low dose of anti-sense is maintained in this region. The anti-sense applications were targeted and timed to block junction formation prior to the periods of elevated expression in the limb, neural tube and face. These times were chosen to optimise the effects of the anti-sense by reducing the expression of new protein rather than being dependent upon the turnover of protein already in the membranes of the cells of the target tissue. Both DB1 and CG1 ODN's reduced expression of a1 connexin protein within two hours in the neural tube and limb bud, dramatic within 4-8 hours and persisted at 18-24 hours and 48 hours in some tissues (data not shown). No down regulation of

a1 connexin protein was evident in any of the controls used. Equally, two other members of the connexin family expressed in the chick embryo, b1 connexin and b2 connexin, were unaffected by the a1 connexin specific anti-sense ODN.

Several parallel controls were run with all of the experiments. These included; DB1 sense, DB1 anti-sense and DB1 sense combined, DB1 chick (which forms stem loop structures with itself), random ODN's CV3, Pluronic gel alone, Pluronic gel with DMSO and PBS alone). None of the controls had a noticeable effect on a1 connexin protein expression.

EXPERIMENT 2

INTRODUCTION

Astrocytes constitute the most abundant cell type in the mammalian brain. They are extensively coupled to one another and to neurons through gap junctions composed predominantly of connexin 43 (Giaume and McCarthy (1996)). Following ischaemia induced or physical brain damage these channels remain open and a spreading wave of depression (initiated by raised interstitial potassium and glutamate and apoptotic signals) is propagated (Cotrina *et al.*, (1998); Lin *et al* (1998)). Waves of increased cytosolic calcium and second messenger molecules such as IP3 are slowly spread via the gap junction channels to neurons beyond the core of the damaged region, resulting in lesion spread in the 24-48 hours following the insult. In this manner, undamaged neighbouring cells are destroyed (Lin *et al.*, 1998), the so-called bystander effect.

This experiment investigates the ability of the formulations of the invention to prevent this bystander effect.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)

TTG TGA TTT ATT TAG TTC GTC TGA TTT C (random control)

METHODS

Oligodeoxynucleotides (ODN's)

Unmodified ODN's were delivered in Pluronic F-127 gel (BASF) in phosphate buffered saline (PBS). Pluronic gel is liquid at low temperatures (0-4°C) and sets at physiological temperatures, and is also a mild surfactant. Unmodified ODN's normally have a half life of approximately 20 min in cells (Wagner, 1994) but the Pluronic gel loading method provides a continual diffusion source, the gel acting as a reservoir Becker *et al.*, (1999)). ODN's specific to connexin 43 were applied, or control random ODN's of similar base composition, at 2mM final concentration. Gel only controls were also carried out. ODN's were 30 mers analysed to show that no hairpin looping or homodimerisation should occur.

Lesioning

Brain lesions were carried out on 250-300 g male Wistar rats. Animals were anaesthetised with 1-2% halothane in oxygen and the head held in a steriotaxic clamp. The region around the lesion site was shaved and the skin over the skull slit in a sagtital plane with a scalpel and pulled back to leave the skull plates clear. A 0.5 mm diameter hole was drilled through the skull plate 3 mm to the right of bregma using an Arlec engraver and a lesion made into the cortex of the brain using a 19G 1½ gauge syringe needle attached to a micrometer stage. The stage allowed accurate directional control and a precise 2 mm penetration depth which kept the lesion within the cortex and well above the corpus callosum.

With the animal prepared, 10ml of ice cold Pluronic F-127 gel (BASF)

containing connexin 43 specific ODN (or a control ODN) was sucked into a precooled 19 G 1½ gauge syringe needle filed off so as to have a flat tip. The syringe needle was attached to a volumetric pipette via a cut down yellow pipette tip. The gel then set in the needle as it warmed to room temperature. The needle with the gel plug at its tip was transferred to a 1 ml syringe containing PBS and a sleeve slipped over the needle shaft so that the needle tip could be lowered into the lesion with the sleeve (coming up against the skull) preventing overpenetration. Gentle pressure on the syringe plunger "popped" the gel plug out of the needle into the lesion. The wound was then treated with hydrogen peroxide to stop bleeding and the skin sutured back into place. Animals were carefully monitored and left until ready for sacrifice 24 hours, 48 hours or 12 days later.

Frozen Sectioning

Animals were sacrificed using Nembutal (pentobarbitone sodium, Virbac) and decapitated. The brains were removed intact and immediately frozen in dry ice snow and stored at -80°C until ready for sectioning. Serial cryosections (30 mm sections) were taken from front to rear (coronal plane), the sections dry mounted onto chrome alum treated slides, and stored for histochemistry or immunohistochemistry at -80°C. The first and last section of each lesion was recorded so that the mid-point sections of the lesion were clearly identified.

Histochemistry

For haemotoxylin and eosin staining sections were hydrated through a descending series of alcohols (absolute, 2 x 95%, 1 x 70% and water) and stained in Gill's haemotoxylin for 4 minutes. The sections were then washed in water, dipped in Scott's water and rewashed in water. They were then stained for 30 seconds in Moore's buffered eosin. The sections were washed once more in water before dehydration through a series of alcohols (2 x 95%, 1 x absolute), 50:50 alcohol:xylol and dipped in xylene. The sections were then mounted using Histomountä mounting medium.

For Nissl staining, sections were dehydrated in an ascending graded series of alochols (75%, 95%, 3 x 100%), five minutes in each, and defatted in xylene for five minutes. The sections were then rehydrated by descending through the same series of alcohols and washed in water. The sections were then placed in a Nissl staining solution (5 ml of a 2% aqueous Cresyl violet stock solution, 90 ml of a 6% glacial acetic acid in water solution, 10 ml of a 1.35% sodium acetate solution) for 10 minutes. The sections were then quickly dehydrated in a series of ascending alcohols for 5 minutes at 75%, then 2 minutes each at 95% and 3 x 100%, three charges of xylene for 10 minutes each. They were then coverslipped with Histomountä mounting medium.

Immunohistochemistry

Frozen sections were first allowed to come back up to room temperature in PBS. They were then permeabilised in methanol for two minutes, rinsed in PBS and transferred to a solution of 0.1M lysine and 0.1% Triton-X 100 in PBS for blocking over 30 min. Two washes in PBS, each of two minutes, followed. PBS was removed and 50 ml per section of primary antibody was applied.

Immunohistochemistry was carried out with primary antibodies against connexin 43, Neuronal-Nuclei (vertebrate specific nuclear protein NeuN) and GFAP (glial fabrillary acidic protein). The following antibodies were used:

Rabbit anti-Cx 43 (Gourdie *et al.*, (1991)) at a concentration of 1:300.
Mouse anti-Cx 43 (Chemicon International, Inc.) at a concentration of 1:100.
Rabbit anti-rat GFAP (DAKO, Z0334), at a concentration of 1:1000.
Mouse anti-Neuronal Nuclei (Chemicon International, Inc.) at a concentration 1:1000.

For connexin and GFAP labelling sections were incubated overnight at 4°C. They were then washed three times 15 minutes in PBS on an orbital shaker. Following this, excess PBS was removed and 50 ml per section of Alexaä 488 anti-

rabbit IgG (Molecular Probes, Oregon, USA) was applied at a concentration of 1:200. For monoclonals and double labelling a CY3 (Chemicon, 132C) anti-mouse secondary antibody was used. Sections were incubated in the dark for two hours at room temperature followed by three washes of 15 minutes in PBS. For mounting excess PBS was removed from the slides and one or two drops of Citifluor (glycerol/PBS solution) anti-fade medium was applied. A coverslip was lowered onto the sections and sealed with nail varnish. For Neuronal-N labelling the secondary antibody was a biotinylated Goat anti-mouse followed by an avidin linked HRP and DAB reaction (Sigma ExtrAvidin or DAKO Quickstain kit).

Imaging and Analysis

Immunofluorescent labelling was carried out using a Leica TCS 4D confocal laser scanning microscope. Double labelled images were subsequently combined using the Leica Combine function or in Adobe Photoshop. Haemotoxylin and eosin, and Nissl stained samples or Neuronal-N labelled sections were captured using a Kontron (Zeiss) Progress 3008 digital camera and lesion areas analysed using MetaMorph (Universal Imaging Corp). Lesion areas were analysed for the middle section of each lesion.

RESULTS

The well documented spread of brain lesions in the first 24 -48 hours after trauma occurred in our control gel experiments and all lesions, controls and antisense treated, tended to spread near the outer edge where the gel is less likely to sit after loading. However, control lesions spread downwards into the corpus callosum and sideways to form ragged, spreading edges (Figures 1 and 2). Examination of Neuronal-N antibody labelled tissues reveals neuronal death occurring well back from the lesion edge, with areas of Nissl staining in which no viable neurons remain. This spread occurs predominantly within 24 hours (Figures 1 and 2), continuing up to 48 hours after lesioning. This is especially apparent in Figure 2 where neuronal death is evident within 24 hours well back from the lesion edge into otherwise

normal looking tissue, and the lesion has spread right down into the corpus callosum. In contrast, the better connexin 43 antisense treated lesions remain confined to the original lesion site and have clearly defined base levels (Figures 3 and 4). Neuronal-N labelling colocalises with Nissl stained tissue and none of the connexin 43 antisense treated lesions spread through the corpus callosum. Neuronal-N labelling shows neuronal survival right up to the edge of the original needle tract lesion. Surviving neurons around these lesions often define sharp boundaries marking the edge of the needle tract (Figures 3 and 5). More tissue remains viable within the lesion itself after antisense treatment; in control lesions cell death leads to tissue loss within the lesion area (compare control lesion in Figure 2 at 24 hours with antisense treated lesions in Figures 3 and 4 at 48 hours).

While antibody labelling of glial fibrillary acidic protein (GFAP) shows some increased astrocyte activation at the edges of lesions, connexin 43 protein levels are clearly reduced at many places along the edge of antisense treated lesions, particularly the basal and medial edges (Figure 6) compared with controls (Figure 7). In some areas the only connexin 43 labelling remaining 24 hours after connexin 43 specific antisense treatment is in blood vessel walls despite raised GFAP levels (Figure 6). In general, connexin 43 labelling around antisense treated lesion collocalises to a much lesser extent with GFAP labelling than in controls in which over half of the connexin 43 labelling is astrocyte related. Other connexin levels (connexins 26 and 32) did not appear to be altered by the connexin 43 specific antisense treatments.

36 animals were lesioned. Cross sectional area (central slice of the lesion volume in a coronal plane) was analysed for 21 animals. The results are shown in Table 2.

Table 2

Cross sectional areas of lesions treated with control and connexin 43 specific oligodeoxynucleotides, left empty, or treated with gel only. Measurements are for animals measured after 24 hours, 48 hours and 12 days. Two sets of figures are included - measurements of the entire lesion, and measurements from 1 mm below the surface. In analysis of the second group the largest DB1 treated lesion (brackets) is excluded as it falls outside 3 standard deviations from the mean for this group. Note that the rat brain does heal (unlike other species) and 12 days lesion measurements do not represent the original extend of lesion spread.

DB1 is anti connexin 43 treated
HB3 is random oligo and appears to be toxic

Entire Lesion: (measurements in square mm)

	24 hours	*48 hours*	*12 days*
DB1	2.42; 3.16; 3.78; 5.57	3.7; 6.05; 2.91; 3.41; 4.53	2.79; 2.86
HB3	7.14	13.19	
Gel/empty	5.04; 4.48	3.96; 3.41; 3.56; 5.91	2.58; 3.3

Lesions from 1 mm down: (this is considered a more accurate measure as all lesions tend to spread at the outer lip indicating that the treatment gel has settled in the bottom of the lesion and/or the outer cortex has been damaged when drilling the skull or inserting the gel loading needle).

	24 hours	*48 hours*	*12 days*
DB1	0.91; 1.13; 2.12; 2.41	(3.38); 0.99; 1.54; 1.44; 1.08	0.47; 1.2
HB3	5.9	5.6	
Gel/empty	3.2; 2.19	1.86; 1.5; 1.68; 2.17	1.07; 1.43

In the final analysis the lesion area from a line 1 mm below the outer cortex edge was measured so as to exclude lesion spread at the outer edge where antisense treatments have little or no effect (owing to gel being injected into and settling at the bottom of lesions). One antisense treated animal falls more than three standard deviations outside the mean for this group and has been excluded. Mean lesion size for antisense treated lesions at 24 and 48 hours was 1.45 mm^2 (+/- 0.55), for controls 2.1 mm^2 (+/- 0.6). The four smallest (of 8 antisense treated and 8 control lesions at 24 and 48 hours) were all connexin 43 antisense treated, with the smallest control lesion 50% larger than these four. This data is also shown in graphical form in Figure 8. By 12 days regeneration occurs in the rat (but not in human brain tissue)

and the limits of lesion spread are not clearly defined.

DISCUSSION

The Pluronic gel plug - antisense ODN method has been used to study the effect of connexin 43 knockdown during astrocytosis which occurs following lesioning of the cerebral cortex of the mammalian brain. In the brain, release of toxins from dying neurons causes what is known as the bystander effect, with the toxins spreading to neighbouring cells through gap junction channels (Lin *et al*, (1998)). Under neurodegenerative conditions, slow release of toxins apparently leads to an upregulation of connexin 43 channels in astrocytes to enable the transport and removal of the toxins to the blood stream. In cases of severe trauma however, this upregulation aids the spread of high toxin levels to neighbouring neurons, killing them. Blocking of the connexin 43 upregulation and knockdown of connexin 43 channels prevents this spread leading to lesions up to 50% smaller in cross sectional area. This has significant implications in the management of ischeamic stroke, treatment of neurodegenerative diseases, and modulation of side effects from surgical intervention.

EXPERIMENT 3

INTRODUCTION

The bystander effect in neural tissues whereby damaged neurons release toxins which spread and kill neighbouring cells is well documented. Experiment 2 shows that this effect can be reduced in the brain using an antisense oligodeoxynucleotide sustained release approach to knockdown the gap junction protein connexin 43.

Another tissue of similar composition to the brain is the spinal cord in which the neural population is supported by populations of glial cells, including astrocytes which are responsible for the neuroprotective effect by removing glutamate and excess calcium from the neural environment. This experiment investigates the ability

of the formulations of the invention to reduce the spread of spinal cord lesions.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)

GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

METHODS

Wistar rats were anaesthetised and their spinal cord exposed. A standard hemisection lesion was then made in the cord and 5 ml of chilled Pluronic gel, containing either antisense or sense ODN's to connexin 43 (5mM) was placed in the lesion. Applications were made blind. The exposed cord was then recovered and the rat returned to its cage. Some animals were sacrificed at 24 hours whereas others were maintained for 12 days and two months in order to determine the extent of neuronal regeneration and the final size of the lesion. For axonal regeneration studies the rats were anaesthetised and their axons severed prior to their entry site to the spinal cord. A pellet of Horse radish peroxidase (HRP) was placed in the cut in order to retrogradely label the axons over a 24 hour period. Next day the rats were sacrificed and their spinal cords removed and fixed in 2% paraformaldehyde. Cords were then processed for cryosectioning and serial longitudinal 8 mm sections were taken through the cords. Sections were then immunostained for either connexins or GFAP along with propidium iodide as a nuclear marker, or processed to reveal the HRP.

RESULTS

At 24 hours post lesion there was a marked difference between the spinal cord lesions treated with connexin 43 sense and antisense ODN's. The sense lesions appeared no different from untreated controls whereas the antisense treated lesions appeared smaller and less inflamed (Figure 9).

At 12 days HRP labelled axons could be seen in both sense and antisense treated cords but in neither case did significant numbers of regenerating axons cross the lesion. However, there was a marked difference in lesion size with the antisense lesion appearing significantly smaller than the sense or untreated lesions.

Two months after lesioning the spinal cords HRP labelling of regenerating axons revealed that they had failed to cross the lesion site in both sense and antisense treatments. Lesion size was significantly smaller in antisense treated cords indicating a significant reduction in secondary neuronal cell death.

DISCUSSION

Using the formulations of the invention, the antisense oligodeoxynucleotide knockdown of connexin 43 significantly reduces the lesion spread which occurs in the first 24-48 hours after spinal cord injury. The knockdown of connexin 43 also reduces inflammation, further aiding in the neuroprotective effect, but there was no change in the ability for neurons to grow back across the lesion site. Thus, antisense treatment with connexin 43 specific oligodeoxynucleotides cannot aid regrowth of damaged neurons, but has a significant neuroprotective effect reducing the spread of the insult.

EXPERIMENT 4

INTRODUCTION

To repair skin wounds a number of cell types, such as fibroblasts, endothelial cells and keratinocytes are activated to proliferate, migrate and lay down extracellular matrix to fill the wound.

Communication and intercellular signalling is a key feature of the wound healing process. Extracellular signalling mechanisms are thought to be the key players though it is also probable that intercellular signalling through the extensive networks of gap junction channels in the skin layers may also have a role. Calcium waves spreading away from injured cells through the epidermis may signal their

damage. In normal wound healing connexin levels start to fall within 6 hours and take up to 6 days to recover. The roles that these changes play are not understood but one theory is that cells are released from their neighbours to divide rapidly, and then junctions reform to coordinate migration into and over the wound site.

This experiment investigates the ability of the formulations of the invention to effect wound healing.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)
GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

METHODS

Neonatal mice, CD1 strain, were anaesthetised with local anaesthetic by spray. A clean incision wound, 2 mm long, was then made along the length of both fore paws with an iridectomy knife. By making the wounds under a dissecting microscope they can be made very reproducible in size. They generally heal in 3-6 days. Carbon powder was dusted into the wounds in order to mark them for subsequent identification of the wound site at late time points - this does not affect the healing in any way. 5 ml of chilled Pluronic gel, containing either Sense or Antisense ODN's was then applied to the wounds. The Pluronic gel is liquid between 0-4°C but sets at higher temperature. Once applied to the wound the gel sets in place and acts as a slow release reservoir for the ODN's as well as a mild surfactant, aiding the penetration of ODN's into the tissue. Application of Sense ODN's was made to one paw and Antisense to the other, alternating left and right between litters. Pups were warmed under a lamp and then returned to their mother. Wounds were examined daily and scored for quality of healing. Representative pups were selected at 1 day, 5 day and 8 day post operation and their forelimbs photographed before the pups were anaesthetised and perfused with 2%

paraformaldehyde. The forelimbs were removed and immersion-fixed in 2% paraformaldehyde overnight and then processed for resin (1 day) or wax (2 days onward) histology.

Inflammation of the wound was assessed 24 hours after wounding. Resin sections through the wound are stained with Toluidine blue to reveal nissl positive cells, neutrophils, which are the first cells to respond to injury. These can also be revealed using neutrophil specific markers.

Cell death and clearance is assessed by Tunel labelling to determine the rate of clearance of apoptotic cells. Macrophage staining was used to show the period of clearing up following cell death. These are carried out days 3-5 post wounding.

Angiogenesis

Granulation is a feature of healing connective tissue and is cased by the invasion of numerous capillaries. Macrophages are known to express potent angiogenic factors such as VEGF. The degree of vascularisation is monitored with antibodies to VEGF receptors, anti-PCAM and anti-flt-1 which are both good blood vessel markers. Contraction of this tissue is brought about by the differentiation of wound fibroblasts into a contractile myofibroblast. After they have pulled the wound together they die apoptotically and are removed by macrophages. These cells can be revealed by smooth muscle actin specific antibodies and their formation and removal followed.

Hyperinnervation

Sensory nerves are very sensitive to the signals released on wounding and show transient sprouting at the sites of adult wounds. However, in neonatal wounds this sprouting is more profuse and results in permanent hyperinnervation. Whilst it is not clear what these signals are it is likely that they are released from inflammatory macrophages. Hyperinnervation is maximal at 7d post wounding and nerve distribution can be revealed using PGP 9.5 antibody against neurofilaments.

Scarring is normally assessed weeks or months after closure of the wound.

However, a reasonable assessment can be made 12 days after wounding. Sections through the wounds are stained with the collagen stain Picrosirus Red and examined on a confocal microscope to determine the collagen density and orientation at the wound site.

RESULTS

1 day

At 24 hours after wounding marked differences were apparent between the sense and antisense treated limbs. Sense treated wounds looked no different from untreated with a normal spectrum of healing grades and rates (Figure 10). Antisense treated limbs were markedly different from the controls, they appeared to be less inflamed and the healing rate was generally faster.

Resin sections of representative limbs stained with a nissl stain revealed significantly less neutrophils cells indicating a less inflamed tissue (Figure 11).

5 days

By days after wounding scabs had started to fall off. At this stage most of the antisense treated wounds appeared to be smaller than the sense treated with either small scabs or less prominent scarring (Figure 12).

8 days

8 days after wounding the limbs had grown hair. Sense treated wounds were still visible being demarcated by a lack of hair around the wound site. Antisense treated wounds were mostly invisible being covered by normal hair growth. This difference in hair growth indicates reduced scarring has occurred in the antisense treated wounds (Figure 13).

CONCLUSIONS

Application of connexin 43 antisense ODN's to a wound has a marked affect on the healing process. The first noticeable effect is a reduction in the inflammation of the wounds which is noticeable in sections which show a much lower inflammatory response in terms of levels of neutrophils. As healing progresses, antisense treated wounds heal faster and with less scarring than control lesions.

This reduction in inflammatory response and subsequent improved healing is possibly owing to reduced neutrophil communication and to a speeding up of natural healing processes. The antisense ODN's can reduce connexin expression in 4-8 hours so they will not have an effect on the initial signalling of wounding but play a role in the secondary signalling events. It is interesting to note that neutrophils which invade in response to the wounding normally express large amounts of connexin 43. It is also possible that they form gap junctions with other cells in the wound and communicate with them. Reduction in this form of communication may result in a reduction of secreted factors from the neutrophils and may reduce cell death in the wound as well as granulation and hyperinnervation. It is also known that under normal conditions connexin protein levels (connexins 26, 31.1 and 43) are reduced in both the epithelial and subdermal layers of wounds starting within 6 hours, and remaining lowered for up to 6 days. The antisense approach may speed up this initial protein reduction by blocking translational processes as protein removal from the membrane is occurring. Certainly, the effects of connexin 43 knockdown immediately following wounding has marked effects on reducing inflammatory levels and increasing healing rates.

EXPERIMENT 5

INTRODUCTION

The inflammation and secondary cell death that follows burning is of major concern. Victims of severe burns over a high percentage of their body often die one

or two days after trauma. This experiment investigates the ability of the formulations of the invention to beneficially affect the burn recovery process.

MATERIALS

Oligodeoxynucleotides were prepared with the following sequences:

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (connexin 43)
GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC (sense control)

METHODS

Reproducible burns are delivered to moistened skin, and Pluronic gel containing antisense ODN's injected subdermal to the burn. A series of burns were made using a soldering iron to the left and right sides of the skull of six newborn mice. The burns on one side of the head were treated with connexin 43-specific ODN in Pluronic gel and those on the other side with sense control ODN in Pluronic gel.

RESULTS

After 24 hours, all six connexin 43 ODN treated burns showed lower levels of inflammation compared with the control burns. These differences were marked (data not shown).

UTILITY

Thus, in accordance with the invention, there are provided formulations by which cell-cell communication can be downregulated in a transient and site-specific manner. The formulations therefore have application in methods of therapy and in cosmetic treatments.

The delivery of the ODN component of the formulation for an extended period (24 hours or longer) is a particular advantage in treating neuronal damage. This is because, in most instances of direct physical neuronal insult, neuronal cell

loss extends well beyond the site of actual injury to the surrounding cells. This secondary neuronal cell loss occurs within 24 hours of the original injury and is mediated by junction gap cell-cell communication. Downregulation of connexin protein expression therefore blocks or at least downregulates communication between the cells and minimises secondary neuronal cell damage.

Equally, in instances of other tissue damage (particularly wounds) the formulations of the invention have been found effective in both promoting the wound healing process, reducing inflammation and in minimising scar formation. The formulations therefore have clear benefit in the treatment of wounds, whether the result of external trauma (including burns) or surgical intervention.

It will further be appreciated that the above description is provided by way of example only and that modifications can be made, both in terms of the specific ODN's and pharmaceutically acceptable carriers or vehicles employed without departing from the scope of the present invention.

Table 1

```
   1 atgggtgact ggagcgcctt aggcaaactc cttgacaagg ttcaagccta ctcaactgct
  61 ggagggaagg tgtggctgtc agtacttttc attttccgaa tcctgctgct ggggacagcg
 121 gttgagtcag cctggggaga tgagcagtct gcctttcgtt gtaacactca gcaacctggt
 181 tgtgaaaatg tctgctatga caagtctttc ccaatctctc atgtgcgctt ctgggtcctg
 241 cagatcatat ttgtgtctgt acccacactc ttgtacctgg ctcatgtgtt ctatgtgatg
 301 cgaaaggaag agaaactgaa caagaaagag gaagaactca aggttgccca aactgatggt
 361 gtcaatgtgg acatgcactt gaagcagatt gagataaaga agttcaagta cggtattgaa
 421 gagcatggta aggtgaaaat gcgagggggg ttgctgcgaa cctacatcat cagtatcctc
 481 ttcaagtcta tctttgaggt ggccttcttg ctgatccagt ggtacatcta tggattcagc
 541 ttgagtgctg tttacacttg caaaagagat ccctgcccac atcaggtgga ctgtttcctc
 601 tctcgcccca cggagaaaac catcttcatc atcttcatgc tggtggtgtc cttggtgtcc
 661 ctggccttga atatcattga actcttctat gttttcttca agggcgttaa ggatcgggtt
 721 aagggaaaga gcgacccttа ccatgcgacc agtggtgcgc tgagccctgc caaagactgt
 781 gggtctcaaa aatatgctta tttcaatggc tgctcctcac caaccgctcc cctctcgcct
 841 atgtctcctc ctgggtacaa gctggttact ggcgacagaa acaattcttc ttgccgcaat
 901 tacaacaagc aagcaagtga gcaaaactgg gctaattaca gtgcagaaca aaatcgaatg
 961 gggcaggcgg gaagcaccat ctctaactcc catgcacagc cttttgattt ccccgatgat
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1141 gagatctag
```

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Lin, J. H., Weigel, H., Cotrina, M. L., Liu, S., Bueno, E., Hansen, A. J., Hansen, T. W., Goldman, S. and Nedergaard, M. (1998). Gap-junction-mediated propogation and amplification of cell injury. *Nature Neurosci.*, 1, 431-432.

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Stein, C. A. (1992): Anti-sense oligodeoxynucleotides - promises and pitfalls, Leukemia 6, 967-974.

Wagner, R. W. (1994): Gene inhibition using anti-sense oligodeoxynucleotides, *Nature*, 372, 333-335.

CLAIMS

1. A formulation for use in therapeutic and/or cosmetic treatment, which formulation comprises:

at least one anti-sense polynucleotide to a connexin protein; together with a pharmaceutically acceptable carrier or vehicle.

2. A formulation according to claim 1, suitable for topical administration.

3. A formulation according to claim 1 or 2, wherein the polynucleotide is an oligodeoxynucleotide.

4. A formulation according to any preceding claim which contains polynucleotides to one connexin protein only.

5. A formulation according to claim 4 wherein said connexin protein is connexin 43, connexin 26, connexin 31.1, connexin 32 or connexin 36.

6. A formulation according to any of claims 1 to 3 which contains polynucleotides to more than one connexin protein.

7. A formulation according to claim 6 in which one of the connexin proteins to which polynucleotides are directed is connexin 43.

8. A formulation according to claim 6 which includes polynucleotides directed to at least two of connexin 26, connexin 31.1, connexin 32, connexin 36 and connexin 43.

9. A formulation according to claim 5, claim 7 or claim 8 in which the polynucleotide to connexin 43 is selected from:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC;
GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC; and
GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT.

10. A formulation according to claim 5 or claim 8 in which the polynucleotide to connexin 26 is:

TCC TGA GCA ATA CCT AAC GAA CAA ATA.

11. A formulation according to claim 5 or claim 8 in which the polynucleotide to connexin 31.1 is:

CGT CCG AGC CCA GAA AGA TGA GGT C.

12. A formulation according to claim 5 or claim 8 in which the polynucleotide to connexin 32 is:

TTT CTT TTC TAT GTG CTG TTG GTG A.

13. A formulation according to any preceding claim in which the pharmaceutically acceptable carrier or vehicle is, or includes, a gel.

14. A formulation according to claim 13 in which the gel is a nonionic polyoxyethylene-polyoxypropylene copolymer gel.

15. A formulation according to any preceding claim which further includes a surfactant or urea to assist with polynucleotide penetration into cells.

16. A method of site-specific downregulation of connexin protein expression for a therapeutic and/or cosmetic purpose which comprises administering a formulation as defined in any one of claims 1 to 15 to a site on or within a patient at which said downregulation is required.

17. A method of reducing neuronal cell death which would otherwise result from a neuronal insult to a specific site in the brain, spinal cord or optic nerve of a patient which comprises the step of administering a formulation as defined in any one of claims 1 to 15 to said site to downregulate expression of connexin protein(s) at and immediately adjacent said site.

18. A method according to claim 17 in which the formulation is administered to reduce neuronal loss due to physical trauma to the brain, spinal cord or optic nerve.

19. A method according to claim 17 or claim 18 in which the formulation is administered in a sufficient amount to downregulate expression of said connexin protein(s) for at least 24 hours post-administration.

20. A method of promoting wound healing in a patient which comprises the step of administering a formulation as defined in any of claims 1 to 15 to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

21. A method according to claim 20 in which the wound is the result of trauma.

22. A method according to claim 21 in which the trauma is a burn.

23. A method according to claim 20 in which the wound is the result of surgery.

24. A method of reducing inflammation as part of treating a wound and/or tissue subjected to physical trauma which comprises the step of administering a formulation as defined in any one of claims 1 to 15 to, or proximate to, said wound or tissue.

25. A method according to claim 24 in which the formulation is administered to reduce inflammation due to physical trauma to the brain, spinal cord or optic nerve.

26. A method of decreasing scar formation in a patient who has suffered a wound which comprises the step of administering a formulation as defined in any one of claims 1 to 15 to said wound to downregulate expression of connexin protein(s) at and immediately adjacent the site of said wound.

27. A method of skin rejuvenation or thickening for a cosmetic or therapeutic purpose which comprises the step of administering, once or repeatedly, a formulation as defined in any one of claims 1 to 15 to the skin surface.

28. A method according to claim 27 wherein said formulation includes polynucleotide directed to connexin 43 and is administered to regulate epithelial basal cell division and growth.

29. A method according to claim 27 wherein said formulation includes polynucleotide directed to connexin 31.1 and is administered to regulate outer layer keratinisation.

30. A method according to any one of claims 27 to 29 wherein the formulation is a cream.

31. The use of at least one anti-sense polynucleotide to a connexin protein in the manufacture of a medicament for downregulating expression of said connexin protein for a therapeutic or cosmetic purpose.

32. The use of claim 31 wherein said medicament is for reducing neuronal cell death which would otherwise result from a neuronal insult.

33. The use of claim 31 wherein said medicament is for promoting wound healing.

34. The use of claim 31 wherein said medicament is for reducing inflammation.

35. The use of claim 31 wherein said medicament is for decreasing scar formation.

36. The use of claim 31 wherein said medicament is for skin rejuvenation for a cosmetic or therapeutic purpose.

FIGURE 1A

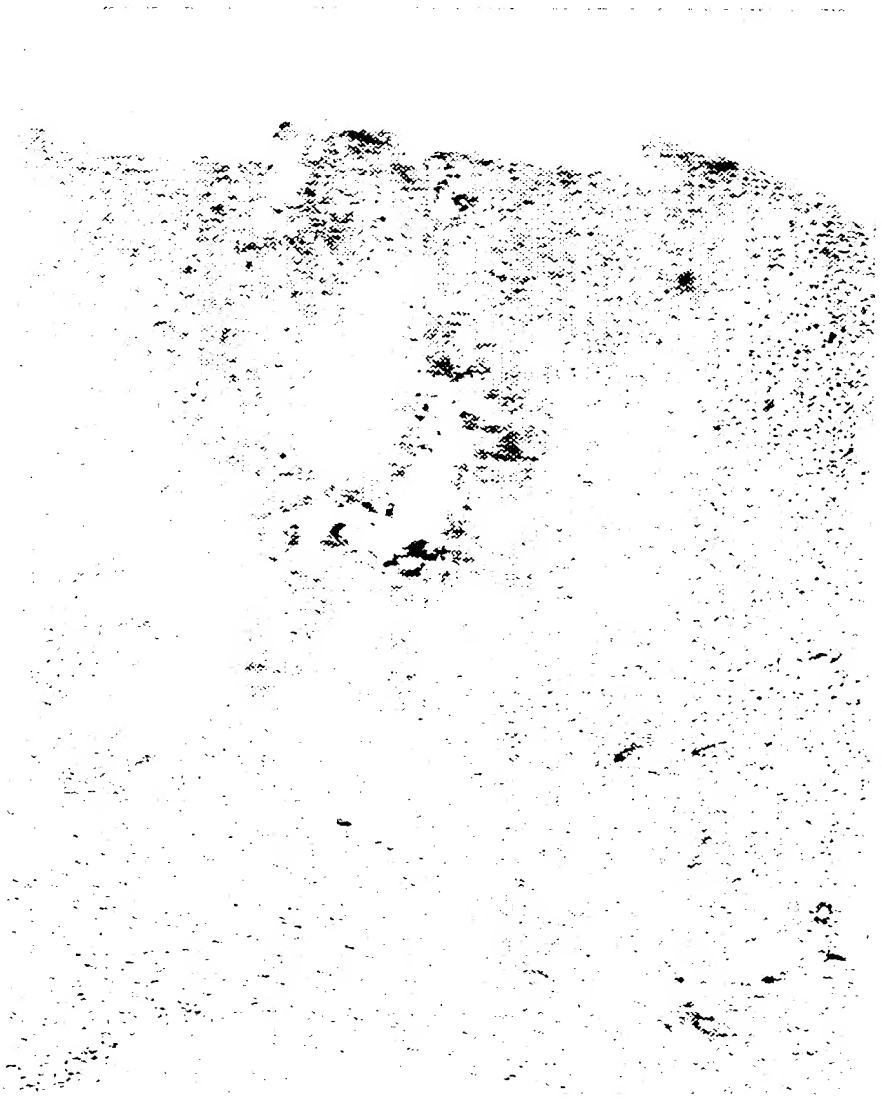

FIGURE 1B

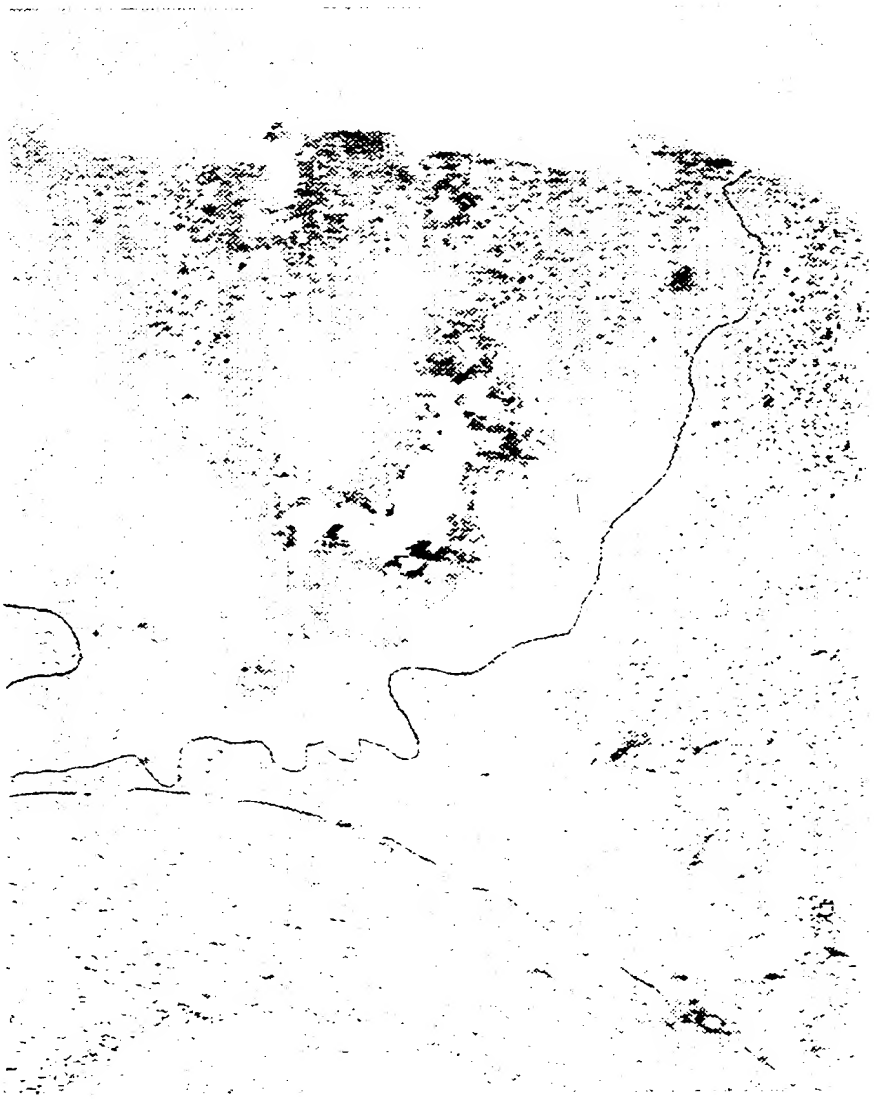

FIGURE 1C

FIGURE 1D

FIGURE 2A

FIGURE 2B

FIGURE 3A

FIGURE 3B

FIGURE 4A

FIGURE 4B

FIGURE 5

FIGURE 6

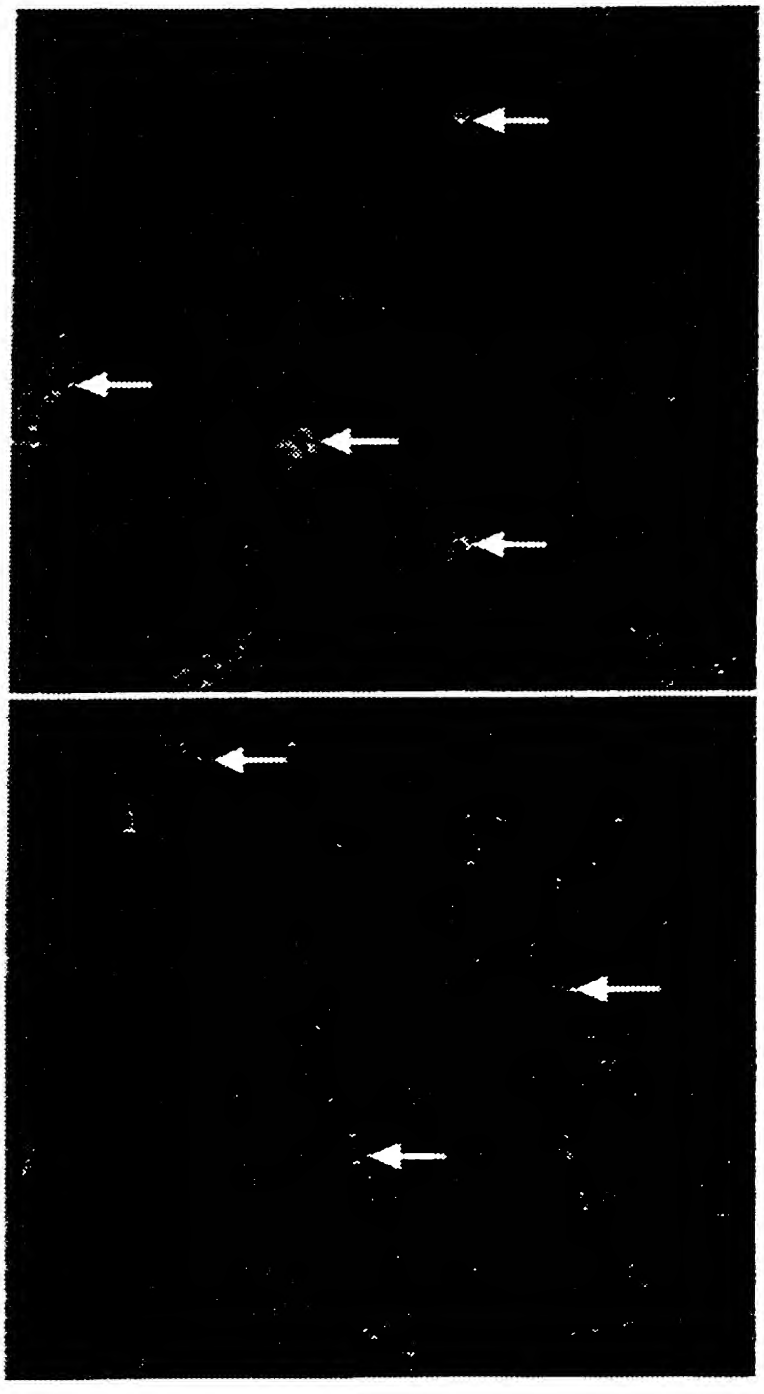

FIGURE 7

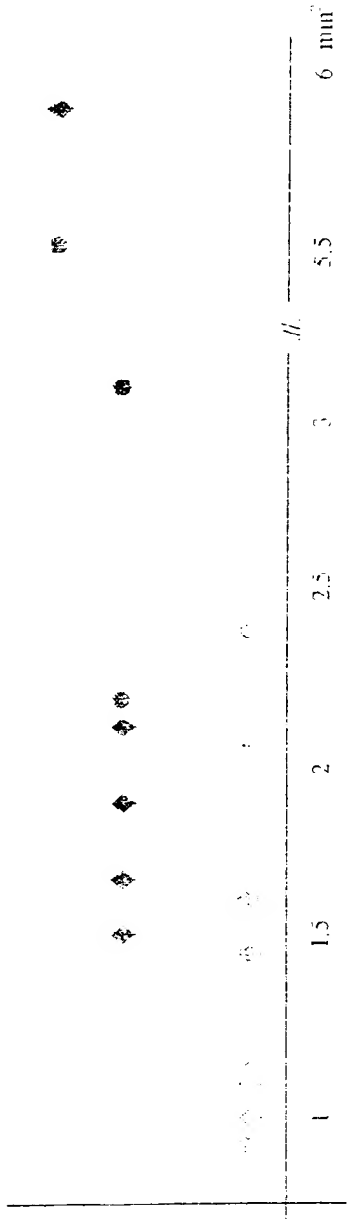
Comparison of lesion areas with different treatments
HB3
Gel only
DB1
1
1.5
2
2.5
3
5.5
6 mm
: 24 hours
: 48hours

FIGURE 8

FIGURE 9

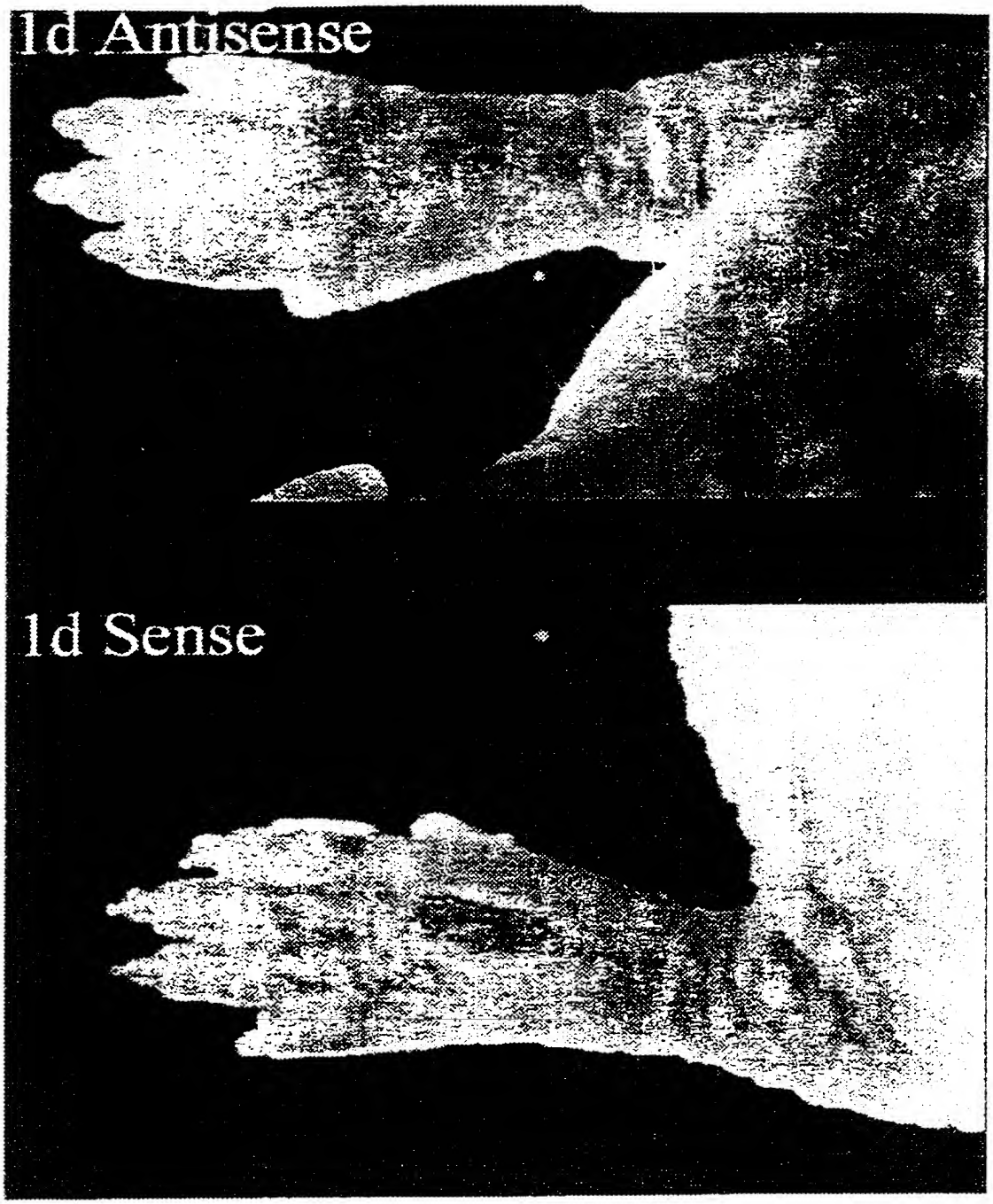

FIGURE 10

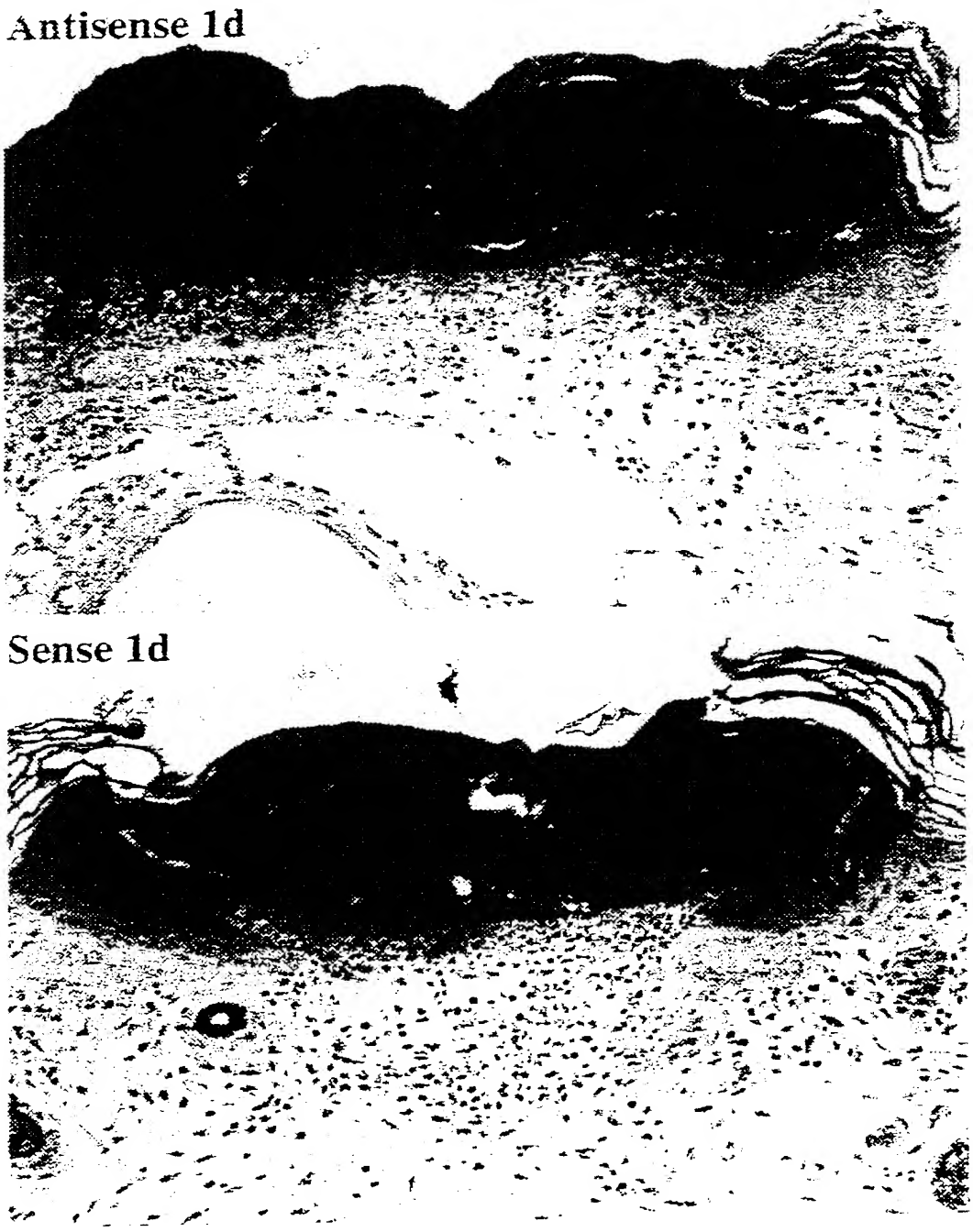

FIGURE 11

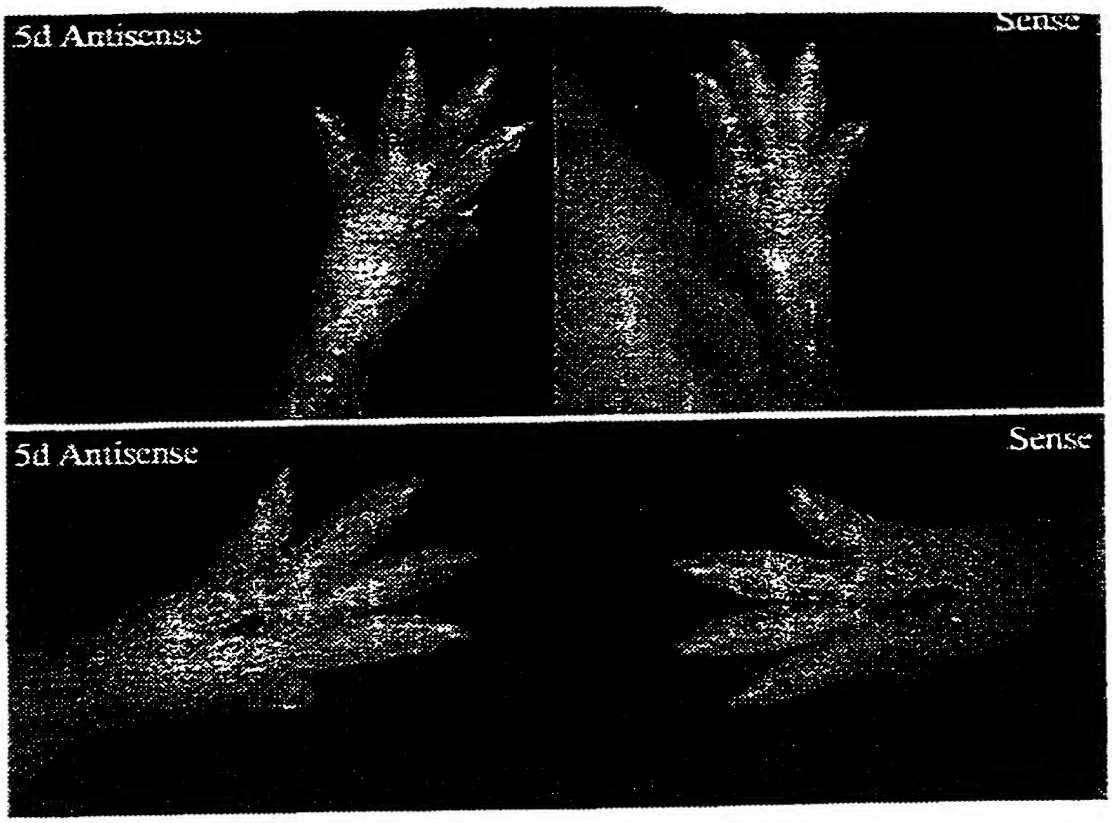

FIGURE 12

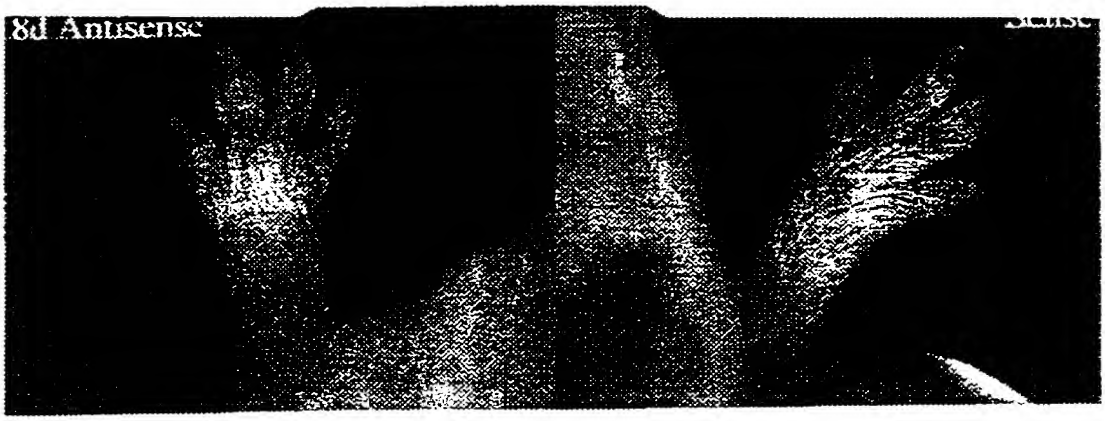

FIGURE 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00238

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 A61K31/70 C12N15/11 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUCH R J ET AL: "Inhibition of gap junctional intercellular communication and enhancement of growth in BALB/c 3T3 cells treated with connexin43 antisense oligonucleotides." MOLECULAR CARCINOGENESIS, (1995 DEC) 14 (4) 269-74. , XP000909565 * see the abstract; page 270 (section "Treatment of cells with ODN' s); page 273, last paragraph * ---	1-5,15, 31-36
A	GOLIGER, JEFFREY A. ET AL: "Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication" MOL. BIOL. CELL (1995), 6(11), 1491-501 , XP000909594 * see the asbstract * ---	1-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2000	19. 06. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Isert, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00238

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 24797 A (HOKE GLENN D ;LEE CHE HUNG (US); BRADLEY MATTHEWS O (US); DYAD PHA) 11 June 1998 (1998-06-11) * see claims 1-22; abstract; page 3, lines 4-18; example 5 (pages 18-19) *	1-36
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN 1995: 31398 on STN, XP002138806 abstract & MOORE ET AL.: "Selective block of gap junction channel expression with connexin-specific antisense oligodeoxynucleotides" AM J PHYSIOLOGY, vol. 265, no. 1, 1994, pages C1371-C1388,	1-5, 31-36
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US STN AN: 1998:379610, XP002138807 abstract & GRAZUL-BILSKA ET AL.: "Transfection of bovine luteal cells with gap junctional protein connexin 43 (Cx43) antisense oligonucleotide affects progesterone secretion" BIOLOGY REPRODUCTION, vol. 58, no. Supp 1, 1998, page 78	1-5, 31-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/00238

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00238

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9824797 A	11-06-1998	AU 1564897 A	29-06-1998
		BR 9612804 A	01-02-2000
		EP 0950060 A	20-10-1999

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

CRESSWELL, Thomas A.
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

KEMP & Co
REC'D 12 APR 2001
Action by.....

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 10.04.2001
Applicant's or agent's file reference N.78821 TAC/GWM	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00238	27/01/2000	27/01/1999

Applicant
UNIVERSITY COLLEGE LONDON et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Exner, K Tel. +49 89 2399-7825

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
N.78821 TAC/GWM		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00238	27/01/2000	27/01/1999

International Patent Classification (IPC) or national classification and IPC
A61K48/00

Applicant
UNIVERSITY COLLEGE LONDON et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/08/2000	10.04.2001

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax +49 89 2399 - 4465

Authorized officer:

Iserl, B

Telephone No. +49 89 2399 8681

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00238

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-36 as originally filed

Claims, No.:

1-36 as originally filed

Drawings, sheets:

1/13-13/13 as originally filed

Sequence listing part of the description, pages:

1-5, filed with the letter of 07.03.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 16-30.

because:

   ☒ the said international application, or the said claims Nos. 16-30 (for industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00238

1. Statement

Novelty (N)	Yes:	Claims	6-14,16-30
	No:	Claims	1-5,15,31-36
Inventive step (IS)	Yes:	Claims	9-12,16-30
	No:	Claims	1-8,13-15,31-36
Industrial applicability (IA)	Yes:	Claims	1-15,31-36
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION III

1). Claims 16-30 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

SECTION V:

2). The following documents (D) cited in the International search report are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1 = Mol. Carcinogenesis, 1995, 14(4): 269-274
D2 = Mol. Biology Cell, 1995, 6 (11): 1491-1501
D3 = WO -A- 98 24797
D4 = Biosis AN: 1995:31398 (STN)
& Am. J. Physiology, 1994, 267(5P1): C1371-1380
D5 = Biosis AN: 1998:379610 (STN)
& Biology Reproduction 1998, 58 (Supp1): 78

Unless indicated otherwise reference is made to the relevant passages emphasized in the search report.

3). Novelty:

Claims 1-5,15,31-36 are not novel in view of documents D1, D4 and D5 disclosing pharmaceutical compositions comprising antisense nucleotides to connexin 43 and the use of the antisense nucleotides in the preparation of a medicament (for example, liposome formulations as described in D1).
Note that the indication of the intended use of a composition is not considered capable of characterising a claim directed to a composition as such. The claims 1 and 31 relate to cosmetic treatment, and further do not specify the intended

therapeutic treatment. Consequently the claims are not considered "first" or "second medical use" claims according to the interpretation used in the EPO, but rather are understood as claims directed to mere (pharmaceutical) compositions. Compositions comprising ODN´s to connexins have been disclosed in D1,D4 and D5.

4). Inventive step

The subject-matter of dependent claims 6-8, 13,14 is not considered to comprise features which are inventive on their own.

The subject-matter of dependent claims 9-12 (novel oligodeoxynucleotides) as well as that of claims 16-30 is considered inventive:

The present application relates to the use of antisense nucleotides to connexins in wound healing. It has been shown in the working examples that antisense oligodeoxynucleotides (ODN´s) to connexin 43 have beneficial effect on the healing process.

Document D3 is considered closest prior art which describes the anti-inflammatory therapeutic use of ODN´s dircted to human adhesion molecules such as ICAM-1. The subject-matter of the present application differs therefrom in that it targets the expression of different proteins, eg. connexin, involved in inflammation.
Thus, the problem to be solved by the present invention could be the finding of alternative ODN´s for use in wound healing. There is an incentive for the skilled person to select ODN´s to connexins in view of D2, which describes the effect of epidermal wounding on connexin 26, 31.1 and 43 expression concluding that intercellular communication (mediated by connexins) is involved in regulating epidermal wound repair. Such ODN formulations have essentially been available, cf. D1, D4 and D5. However, the skilled man would not have expected the effects observed, as D1 points to limitations of ODN´s when used in vivo.

5). Industrial applicability

5.1 For the assessment of the present claims 16-30 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

5.2 The claims 1-15,31-36 relate to pharmaceutical products and their manufacture being industrially applicable under Article 33(4) PCT.

SECTION VII

6). Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D5 is not mentioned in the description, nor are these documents identified therein.

SECTION VIII

7). The terms "oligonucleotides" and "polynucleotides" used in the claims are not clear in the light of the ambiguous definition given at page 9, lines 23-30. Similarly the wording used in claim 3 seems to be contradictory ("the polynucleotide is an oligodeoxynucleotide).

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)*

Box No. I TITLE OF INVENTION

FORMULATIONS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

UNIVERSITY COLLEGE LONDON
GOWER STREET
LONDON, WC1E 6BT
UNITED KINGDOM

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: UNITED KINGDOM

State *(that is, country)* of residence: UNITED KINGDOM

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BECKER, DAVID LAURENCE
19 LAPWING WAY
ABBOTS LANGLEY
HERTFORDSHIRE
UNITED KINGDOM

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: UNITED KINGDOM

State *(that is, country)* of residence: UNITED KINGDOM

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

CRESSWELL, THOMAS ANTHONY
J.A. KEMP & CO.,
14 South Square,
Gray's Inn,
London, WC1R 5LX,
United Kingdom.

Telephone No.
+44 171 405 3292

Facsimile No.
+44 171 242 8932

Teleprinter No.
23676

☐ **Adress for correspondence:** Mark this check-box where no agent or common representative is has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No 2

Continuation of Box No. III **FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS**

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GREEN, COLIN RICHARD
3/4 CRESCENT ROAD
EPSOM
AUCKLAND 3
NEW ZEALAND

This person is:

☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NEW ZEALAND

State *(that is, country)* of residence: NEW ZEALAND

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [X] AP **ARIPO Patent**: **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] EA **Eurasian Patent**: **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] EP **European Patent**: **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] OA **OAPI Patent**: **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)*

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] AE United Arab Emirates
- [X] AL Albania
- [X] AM Armenia
- [X] AT Austria
- [X] AU Australia
- [X] AZ Azerbaijan
- [X] BA Bosnia and Herzegovina
- [X] BB Barbados
- [X] BG Bulgaria
- [X] BR Brazil
- [X] BY Belarus
- [X] CA Canada
- [X] CH and LI Switzerland and Liechtenstein
- [X] CN China
- [X] CU Cuba
- [X] CZ Czech Republic
- [X] DE Germany
- [X] DK Denmark
- [X] EE Estonia
- [X] ES Spain
- [X] FI Finland
- [X] GB United Kingdom
- [X] GD Grenada
- [X] GE Georgia
- [X] GH Ghana
- [X] GM Gambia
- [X] HR Croatia
- [X] HU Hungary
- [X] ID Indonesia
- [X] IL Israel
- [X] IN India
- [X] IS Iceland
- [X] JP Japan
- [X] KE Kenya
- [X] KG Kyrgyzstan
- [X] KP Democratic People's Republic of Korea
- [X] KR Republic of Korea
- [X] KZ Kazakhstan
- [X] LC Saint Lucia
- [X] LK Sri Lanka
- [X] LR Liberia
- [X] LS Lesotho
- [X] LT Lithuania
- [X] LU Luxembourg
- [X] LV Latvia
- [X] MD Republic of Moldova
- [X] MG Madagascar
- [X] MK The former Yugoslav Republic of Macedonia
- [X] MA Morocco
- [X] MN Mongolia
- [X] MW Malawi
- [X] MX Mexico
- [X] NO Norway
- [X] NZ New Zealand
- [X] PL Poland
- [X] PT Portugal
- [X] RO Romania
- [X] RU Russian Federation
- [X] SD Sudan
- [X] SE Sweden
- [X] SG Singapore
- [X] SI Slovenia
- [X] SK Slovakia
- [X] SL Sierra Leone
- [X] TJ Tajikistan
- [X] TM Turkmenistan
- [X] TR Turkey
- [X] TT Trinidad and Tobago
- [X] UA Ukraine
- [X] UG Uganda
- [X] US United States of America
- [X] TZ Tanzania
- [X] UZ Uzbekistan
- [X] VN Viet Nam
- [X] YU Yugoslavia
- [X] ZA South Africa
- [X] ZW Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- [X] CR Costa Rica
- [X] DM Dominica

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information, in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available, in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country of residence if no State of residence is indicated below);*

*(ii) if in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked, in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if in Box No. II or in any of the sub-boxes of Box No. III **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**, in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**, in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if in Box No. V, the name of any State (or OAPI) is accompanied by the indication "**patent of addition**," or "**certificate of addition**," or if, in Box No. V, the name of the United States of America is accompanied by an indication "**continuation**" or "**continuation-in-part**", in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**, in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if in Box No. VI, **the earlier application is an ARIPO application**, in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

GOLDIN, Douglas Michael; ELLIS-JONES, Patrick George Armine; BARLOW, Roy James; SENIOR, Alan Murray; BENTHAM, Stephen; AYERS, Martyn Lewis Stanley; WOODS, Geoffrey Corlett; CRESSWELL, Thomas Anthony; SEXTON, Jane Helen; NICHOLLS, Michael John, MARSHALL Monica Anne; WEBB, Andrew John; KEEN, Celia Mary; PRICE, Nigel John King; IRVINE, Jonquil Claire; LEEMING, John Gerard; DUCKWORTH, Timothy John; MCCLUSKIE, Gail Wilson; WRIGHT, Simon Mark, CURWEN, Julian Charles Barton, CLEEVE, James Harold Findlay; SMITH, Samuel Leonard; BENSON, John Everett, CAMPBELL Patrick John; MERRYWEATHER, Colin Henry; DUCKETT, Anthony Joseph BENTHAM, Andrew; and ROQUES, Sarah Elizabeth; SRINIVASAN, Ravi Chandran; TYSON, Robin Edward and BURNSIDE Ivan John of: J.A. KEMP & CO , 14 South Square, Gray's Inn, London, WC1R 5LX, United Kingdom.

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box

Filing date of earlier application (day month year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 27.01.99	333928	NEW ZEALAND		
item (2) 07.10.99	500136	NEW ZEALAND		
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s) _____

* *Where the earlier application is an ARIPO application it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(iii)). See Supplemental Box*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / EPO

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(for regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 5
description (excluding sequence listing part)	: 36
claims	: 4
abstract	: 1
drawings	: 18
sequence listing part of description	:
Total number of sheets	: 64

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application:

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

McCLUSKIE, GAIL WILSON

For receiving Office use only

1. Date of actual receipt of the purported international application

2. Drawings:
   ☐ received
   ☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA

6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau

This sheet is not part of and does not count as a sheet of the international application

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No

Date stamp of the receiving Office

Applicant's or agent's file reference: N 78821 - TAC/GWM

Applicant: UNIVERSITY COLLEGE LONDON

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £ 55 [T]

2. SEARCH FEE . . . . . £ 638 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 64 sheets.

first 30 sheets . . . . . £ 264 [b1]

34 (remaining sheets) x £6 (additional amount) = £ 204 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . £ 468 [B]

Designation Fees

The international application contains ALL designations.

8 (number of designation fees payable *(maximum 10)*) x £56 (amount of designation fee) = £ 448 [D]

Add amounts entered at B and D and enter total at I . . . . . £ 916 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . [P]

5. TOTAL FEES PAYABLE . . . . . £1.609 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☐ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☒ cheque	☐ cash	☐ other *(specify)*
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO _____ ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No _____ Date *(day month year)* _____ Signature _____

See Notes to the fee calculation sheet